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Synthesis and *in vitro* anticancer activity of *N*-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (AZT)

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Abstract

In this study, a series of novel *N*-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (AZT) were synthesized using two methods. The synthesized phosphoramidates **7a**–**e** were evaluated for their cytotoxic activity in three human cancer cell lines (cervical cancer (HeLa), nasopharyngeal cancer (KB), and breast cancer (MCF-7)) and a normal dermal fibroblast cell line (HDF) using sulforhodamine B assay. Among the synthesized phosphoramidates, the highest cytotoxic activity was demonstrated by phosphoramidate **7d** with the *N*-*n*-propyl substituent in all the examined cancer cell lines, and its activity was found to be about two fold higher than that of the parent nucleoside (AZT). Phosphoramidate **7d** showed not only a high cytotoxic activity against cancer cell lines but also a low toxicity against normal fibroblast cells; its selectivity index was >3 for all the investigated cancer cell lines. A slightly lower cytotoxic activity was shown by phosphoramidates **7a**, **7b**, and **7e**, whereas phosphoramidate **7c** with the *N*-(2,2,2-trifluoroethyl) substituent exhibited the least cytotoxic activity in all the cell lines used.

Key words: *N*-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine, cytotoxic activity, human cancer cell lines – HeLa, KB, MCF-7, normal human cell line – HDF

Introduction

A number of nucleoside analogs have been found to possess beneficial properties that enable their use as antiviral (De Clercq and Li, 2016) and anticancer (Shelton et al., 2016) therapeutics. In particular, the first approved anti-HIV (human immunodeficiency virus) drug, 3′-azido-3′-deoxythymidine (AZT, zidovudine) played an important role in the fight against acquired immunodeficiency syndrome (AIDS) in the initial outbreak of its pandemic. Some reports have indicated the use of AZT as an antitumor agent in combination with either 5-fluorouracil or methotrexate in the treatment of advanced colon cancer (Brunetti et al., 1990; Tosi et al., 1992; Darnowski and Goulette, 1994). Furthermore, a study demonstrated the potent growth inhibitory effect of AZT in cultured human breast cancer cells (Wagner et al., 1997). It is assumed that AZT exerts its anticancer action by undergoing intracellular conversion into 5′-triphosphate (via the corresponding mono- and diphosphate). The resulting 5′-triphosphate acts as a competitive inhibitor of DNA polymerases and as a chain terminator of the growing DNA strand because it lacks 3′-hydroxyl group. Due to their potential anticancer effect, development of AZT prodrugs (pronucleotides), which can easily cross the lipid-rich cell membrane and undergo chemical or enzymatic hydrolysis into 5′-monophosphate, has become an important area of research (Wagner et al., 2000; Hecker and Erion, 2008; Pradere et al., 2014). 5′-monophosphate of AZT released inside the cell would require only a second and third phosphorylation

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for the conversion to 5′-triphosphate of AZT. The 5′-monophosphate of AZT cannot be employed as a prodrug as it is negatively charged at physiological pH and consequently too polar to cross the cell membrane. Thus far, many AZT monophosphate prodrugs were synthesized and evaluated for antiviral activity (Romanowska et al., 2011; Pradere et al., 2014). However, only a few have been tested for anticancer activity (Celewicz et al., 2011); the *N*-amino acid phosphoramidate monoesters of AZT investigated by Wagner and coworkers (Iyer et al., 2000) are one among them.

This paper reports the synthesis of *N*-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (**7a**–**e**) and evaluation of their cytotoxic activity in three human cancer cell lines (cervical cancer (HeLa), nasopharyngeal cancer (KB), and breast cancer (MCF-7)) and a normal dermal fibroblast cell line (HDF).

Materials and methods

Chemistry

The nuclear magnetic resonance (NMR) spectra of the synthesized phosphoramidates were recorded using a Varian-Gemini 300 Hz spectrometer. Chemical shifts (δ) were measured in ppm relative to the tetramethylsilane peak. The ^{31}P NMR spectra of the phosphoramidates were recorded using 85% phosphoric (V) acid in D_2O as an external standard in coaxial inner tube. The mass spectra of the compounds were recorded using a Waters Micromass ZQ electrospray mass spectrometer. After synthesis, the phosphoramidates were purified by high-performance liquid chromatography (HPLC) using a Waters chromatograph equipped with a Waters 996 UV-Vis photodiode array detector. All the chemical reagents used in this study were purchased from Sigma–Aldrich (now Merck). Triethylammonium 3′-azido-3′-deoxythymidine 5′-*O*-phosphite (**6**) was prepared according to a previously described procedure (Iyer et al., 2000).

General procedures for the synthesis of target compounds 7a–e

Method 1

Triethylammonium 3′-azido-3′-deoxythymidine 5′-*O*-phosphite (**6**) (200 mg, 0.462 mmol) was first dissolved in dry pyridine (8 ml). The mixture was rendered anhydrous by adding 4A molecular sieves (0.3 g) and then treated with trimethylsilyl chloride (176 ml, 1.39 mmol) in the presence of argon. After 5 min, a solution of iodine (176 mg, 0.694 mmol) in pyridine (5 ml) was added dropwise to the reaction mixture until the color of the mixture changed from yellow to reddish brown. Then, an appropriate amine (1.386 mmol) or a solution of amine hydrochloride (1.386 mmol) in a mixture of pyridine (5 ml) and triethylamine (0.4 ml) was added to the reaction mixture. After stirring for 20 min, the reaction mixture was concentrated under reduced pressure. The residue obtained was dissolved in water (10 ml). Then, triethylamine (2 ml) was added and the resulting solution was evaporated under reduced pressure. The residue obtained was again dissolved in water (3 ml) and filtered through a Millipore HA (0.45 μm) filter. The filtered solution was chromatographed on a Waters X-Terra™ MS C_{18} (19 × 100 mm, 5 μm) reverse-phase HPLC column using water–acetonitrile (9 : 1, v/v) mixture as an eluent to obtain pure **7a**–**e** (yield 54–63%).

Method 2

To a solution of phosphorus oxychloride (0.209 ml, 344 mg, 2.245 mmol) in acetonitrile (5 ml), 1,2,4-triazole (512 mg, 7.410 mmol) was added, followed by triethylamine (0.950 ml, 6.735 mmol). After addition, the reaction mixture was stirred for 15 min. Then, a solution of 3′-azido-3′-deoxythymidine (500 mg, 1.870 mmol) in pyridine (2.5 ml) was added and the reaction mixture was stirred at room temperature for 40 min. Subsequently, an appropriate amine (2.245 mmol) or a mixture of amine hydrochloride (2.245 mmol) and triethylamine (2.245 mmol) was added, and the reaction mixture was stirred for 40 min. After stirring, the reaction mixture was evaporated under reduced pressure. The residue obtained was dissolved in water (20 ml). Then, triethylamine (4 ml) was added and the resulting solution was evaporated under reduced pressure. The residue obtained was again dissolved in water (3 ml) and filtered through a Millipore HA (0.45 μm) filter. The filtered solution was chromatographed on a Waters X-Terra™ MS C_{18} (19 × 100 mm, 5 μm) reverse-phase HPLC column using water–acetonitrile (9 : 1, v/v) mixture as an eluent to obtain pure **7a**–**e** (yield 35–41%).

3′-azido-3′-deoxythymidyn-5′-yl (N-methyl)phosphoramidate triethylammonium salt – 7a

^{1}H NMR (DMSO-d_6) δ: 1.16 (t, 9H, J = 7.1 Hz, N-(C-CH_3)$_3$), 1.83 (d, 3H, J = 1.1 Hz, 5-CH_3), 2.23–2.46 (m, 5H, H-2″, H-2′, N-CH_3), 2.94 (q, 6H, J = 7.1 Hz, N-(CH_2-C)$_3$), 3.82 (m, 4H, H-5″, H-5′, P-NH, Et_3NH), 3.96 (m,

1H, H-4′), 4.48 (m, 1H, H-3′), 6.14 (pseudo t, 1H, J = 6.6 Hz, H-1′), 7.82 (d, 1H, J = 1.1 Hz, H-6), 11.21 (bs, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 8.55 (CH_3 of Et_3NH), 12.08 (5-CH_3), 27.96 (d, J_{C-P} = 6.8 Hz, N-CH_3), 36.19 (N-CH_2), 44.96 (C-2′), 60.86 (C-3′), 63.36 (d, J_{C-P} = 7.0 Hz, C-5′), 83.02 (C-1′), 83.48 (d, J_{C-P} = 6.8 Hz, C-4′), 109.88 (C-5), 136.16 (C-6), 150.54 (C-2), 163.86 (C-4). ^{31}P NMR (DMSO-d_6) δ: 10.00. MS-ESI *m/z*: 359 $[C_{11}H_{16}N_6O_6P]^-$.

3′-azido-3′-deoxythymidyn-5′-yl (N-ethyl)phosphoramidate triethylammonium salt – 7b

^{1}H NMR (DMSO-d_6) δ: 0.98 (t, 3H, J = 7.1 Hz, P-N-C-CH_3), 1.16 (t, 9H, J = 7.1 Hz, N-(C-CH_3)$_3$), 1.82 (d, 3H, J = 1.1 Hz, 5-CH_3), 2.26 (m, 1H, H-2″), 2.38 (m, 1H, H-2′), 2.74 (q, 2H, J = 7.1 Hz, P-N-CH_2), 2.96 (q, 6H, J = 7.1 Hz, N-(CH_2-C)$_3$), 3.83 (m, 4H, H-5″, H-5′, P-NH, Et_3NH), 3.96 (m, 1H, H-4′), 4.48 (m, 1H, H-3′), 6.14 (pseudo t, 1H, J = 6.6 Hz, H-1′), 7.81 (d, 1H, J = 1.1 Hz, H-6), 11.40 (bs, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 8.56 (CH_3 of Et_3NH), 12.13 (5-CH_3), 17.46 (d, J_{C-P} = 6.9 Hz, CH_3 of P-N-Et), 36.02 (d, J_{C-P} = 7.0 Hz, P-N-CH_2), 36.16 (CH_2 of Et_3NH), 44.94 (C-2′), 60.80 (C-3′), 63.34 (d, J_{C-P} = 7.4 Hz, C-5′), 82.78 (C-1′), 83.37 (d, J_{C-P} = 6.9 Hz, C-4′), 109.72 (C-5), 135.93 (C-6), 150.28 (C-2), 163.57 (C-4). ^{31}P NMR (DMSO-d_6) δ: 6.95. MS-ESI *m/z*: 373 $[C_{12}H_{18}N_6O_6P]^-$.

3′-azido-3′-deoxythymidyn-5′-yl (N-2,2,2-trifluoroethyl)phosphoramidate triethylammonium salt – 7c

^{1}H NMR (DMSO-d_6) δ: 1.19 (t, 9H, J = 7.1 Hz, N-(C-CH_3)$_3$), 1.82 (d, 3H, J = 1.1 Hz, 5-CH_3), 2.27 (m, 1H, H-2″), 2.40 (m, 1H, H-2′), 3.02 (q, 6H, J = 7.1 Hz, N-(CH_2-C)$_3$), 3.41 (q, 2H, J = 9.60 Hz, P-N-CH_2), 3.64 (bs, 2H, P-NH, Et_3NH), 3.83 (m, 2H, H-5″, H-5′), 3.95 (m, 1H, H-4), 4.46 (m, 1H, H-3′), 6.14 (pseudo t, 1H, J = 6.6 Hz, H-1), 7.77 (d, 1H, J = 1.1 Hz, H-6), 11.45 (bs, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 8.43 (CH_3 of Et_3NH), 12.04 (5-CH_3), 36.10 (CH_2 of Et_3NH), 43.45 (d, J_{C-P} = 7.0 Hz, P-N-CH_2), 45.19 (C-2′), 60.84 (C-3′), 63.60 (d, J_{C-P} = 7.8 Hz, C-5′), 82.76 (C-1′), 83.42 (d, J_{C-P} = 7.1 Hz, C-4), 109.74 (C-5), 130.10 (m, CF_3), 135.90 (C-6), 150.30 (C-2), 163.55 (C-4). ^{19}F NMR (DMSO-d_6) δ: -71.74 (t, 3F, J_{H-F} = 9.6 Hz, CF_3). ^{31}P NMR (DMSO-d_6) δ: 5.30. MS-ESI *m/z*: 427 $[C_{12}H_{15}F_3N_6O_6P]^-$.

3′-azido-3′-deoxythymidyn-5′-yl (N-n-propyl)phosphoramidate triethylammonium salt – 7d

^{1}H NMR (DMSO-d_6) δ: 0.79 (t, 3H, J = 7.3 Hz, P-N-C-C-CH_3), 1.13 (t, 9H, J = 7.1 Hz, N-(C-CH_3)$_3$), 1.34 (sextet, 2H, J = 7.3 Hz, P-N-C-CH_2-C), 1.83 (d, 3H, J = 1.0 Hz, 5-H_3), 2.30 (m, 1H, H-2″), 2.44 (m, 1H, H-2′), 2.66 (q, 6H, J = 7.1 Hz, N-(CH_2-C)$_3$), 2.86 (q, 2H, J = 7.1 Hz, P-N-CH_2), 3.50 (bs, 2H, P-NH, Et_3NH), 3.80 (m, 2H, H-5″, H-5′), 3.96 (m, 1H, H-4′), 4.50 (m, 1H, H-3′), 6.14 (pseudo t, 1H, J = 6.7 Hz, H-1′), 7.86 (d, 1H, J = 1.0 Hz, H-6), 11.20 (bs, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 9.01 (CH_3 of Et_3NH), 11.38 (CH_3 of N-n-Pr), 12.04 (5-CH_3), 20.46 (P-N-C-CH_2), 24.83 (d, J_{C-P} = 7.7 Hz, P-N-CH_2), 36.22 (CH_2 of Et_3NH), 43.56 (C-2), 61.04 (C-3′), 63.34 (d, J_{C-P} = 7.2 Hz, C-5′), 83.13 (C-1), 83.48 (d, J_{C-P} = 6.8 Hz, C-4′), 109.86 (C-5), 136.22 (C-6), 150.54 (C-2), 163.85 (C-4). ^{31}P NMR (DMSO-d_6) δ: 7.04. MS-ESI *m/z*: 387 $[C_{13}H_{20}N_6O_6P]^-$.

3′-azido-3′-deoxythymidyn-5′-yl (N-piperidinyl)phosphoramidate triethylammonium salt – 7e

^{1}H NMR (DMSO-d_6) δ: 1.38 (t, 9H, J = 7.1 Hz, N-(C-CH_3)$_3$), 1.47 (m, 2H, P-N-C-C-CH_2), 1.66 (m, 4H, P-N-C-CH_2), 1.83 (d, 3H, J = 1.1 Hz, 5-CH_3), 2.27 (m, 1H, H-2″), 2.40 (m, 1H, H-2′) 2.90 (m, 10H, P-N-CH_2, N-CH_2 of Et_3NH), 3.50 (bs, 2H, P-NH, Et_3NH), 3.82 (m, 2H, H-5″, H-5′) 3.94 (m, 1H, H-4′) 4.49 (m, 1H, H-3′) 6.13 (pseudo t, 1H, J = 6.6 Hz, H-1′) 7.83 (d, 1H, J = 1.1 Hz, H-6), 11.04 (bs, 1H, 3-NH). ^{13}C NMR (DMSO-d_6) δ: 8.58 (CH_3 of Et_3NH), 12.07 (5-CH_3), 22.28 (P-N-C-C-CH_2), 24.71 (d, J_{C-P} = 6.3 Hz, P-N-C-CH_2), 26.18 (d, J_{C-P} = 7.9 Hz, P-N-CH_2), 36.28 (CH_2 of Et_3NH), 43.54 (C-2′), 60.85 (C-3′), 63.52 (d, J_{C-P} = 7.5 Hz, C-5′), 83.15 (C-1′), 83.46 (d, J_{C-P} = 6.4 Hz, C-4′), 109.64 (C-5), 135.98 (C-6), 150.26 (C-2), 163.53 (C-4). ^{31}P NMR (DMSO-d_6) δ: 6.65. MS-ESI *m/z*: 413 $[C_{15}H_{22}N_6O_6P]^-$.

Biological evaluation

Cell cultures

The cell lines HeLa, KB, MCF-7, and HDF (fetal) were obtained from The European Collection of Cell Cultures supplied by Sigma-Aldrich (now Merck). KB cells (human nasopharyngeal carcinoma cell line) were cultured in RPMI (Roswell Park Memorial Institute medium) 1640, MCF-7 cells (human breast cancer cell line) were cultured in DMEM (Dulbecco's modified Eagle's medium), and HeLa cells (human cervical cancer cell line) were cultured in MEM (Eagle's minimal essential medium). All the mentioned media were supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin solution. The cells were placed in an incubator at 37 °C in a humidified atmosphere (90% rela-

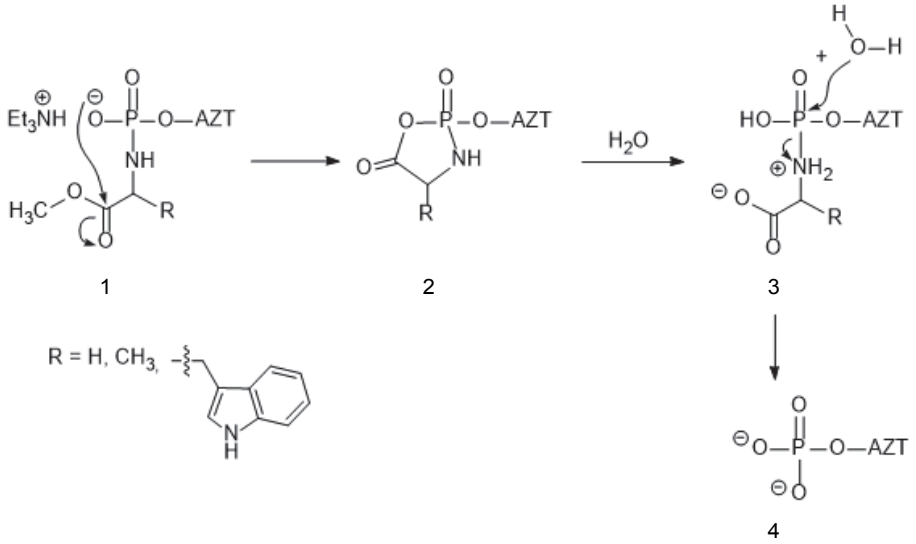

Fig. 1. Proposed decomposition mechanism of N-amino acid methyl ester phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (AZT)

tive humidity, RH) in the presence of 5% CO_2. The optimal plating density of the cells was determined to be 5×10^4.

In vitro cytotoxicity assay

The protein-staining sulforhodamine B (SRB, Sigma-Aldrich) microculture colorimetric assay, developed by the National Cancer Institute (USA) for *in vitro* antitumor screening, was performed to estimate the cell number. This assay provides a sensitive index of the total cellular protein content, which is linear to cell density (Skehan et al., 1990). The assay was carried out as follows: First, the monolayer cell culture was trypsinized, and the cell count was adjusted to 5×10^4. Then, 100 µl of the cell suspension in a growth medium (containing approximately 10 000 cells) was added to each well of a 96-well microtiter plate. Each test compound was dissolved in dimethyl sulfoxide (DMSO) (50 µl, containing 10% of water), the content of DMSO was maintained below 0.1% as this concentration was found to be nontoxic to the cell lines. After 24 hrs of incubating the cells on the microtiter plate, when a partial monolayer had formed, the supernatant was carefully removed, and 100 µl of the test compound at six different concentrations (0.1, 0.2, 1, 2, 10, and 20 µM) was added to the cells. The cells were exposed to the compounds for 72 h at 37 °C in a humidified atmosphere (90% RH) in the presence of 5% CO_2. Then, 25 µl of 50% trichloroacetic acid was added to the wells and the plates were incubated for 1 h at 4 °C. Following incubation, the plates were washed with distilled water to remove any traces of medium and air dried. The air-dried plates were stained with 100 µl of 0.4% SRB (prepared in 1% acetic acid) and left for 30 min at room temperature. The unbound dye was removed by washing five times with 1% acetic acid, and the plates were air dried overnight. For determination of optical density, the protein-bound dye was dissolved in 100 µl of 10 mM unbuffered Tris base (pH 10.5) and the absorbance was read at 490 nm. All the cytotoxicity experiments were performed in triplicate. Cell survival was measured as the percentage absorbance of treated cells compared to the absorbance of control (nontreated cells). Cytarabine (Sigma-Aldrich) was used as the internal standard for this assay.

Result and discussion

This study focused on the synthesis and evaluation of the anticancer activity of *N*-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine. The *N*-amino acid methyl ester phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (i.e. glycine, alanine, and tryptophan derivatives), which were previously synthesized by Wagner and coworkers (Iyer et al., 2000), were found to be unstable in aqueous solutions; their half-life was only in the range of 2–4 hrs. This instability might be because the phosphoramidates with *N*-amino acid methyl ester substituents (**1**) are prone to decomposition due to the formation of mixed phosphoric–carboxylic acid anhydride

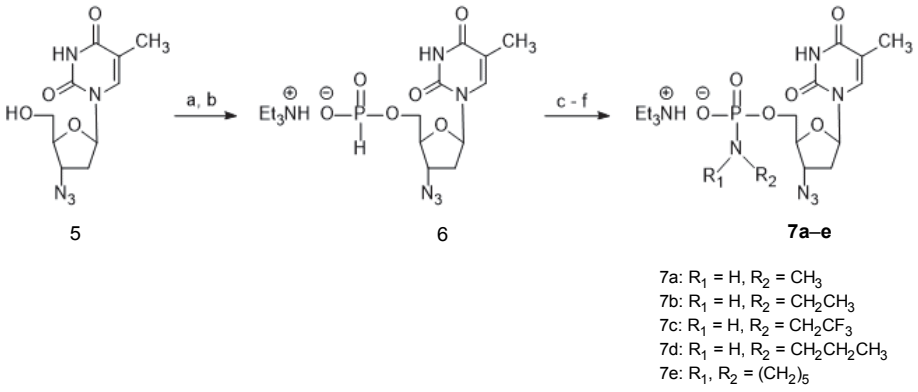

Fig. 2. Synthesis of N-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (**7a**–**e**) via 5′-H-phosphonate monoester of 3′-azido-3′-deoxythymidine (**6**); reagents and conditions: (a) diphenylphosphite; Py; (b) Et_3N, H_2O; (c) TMSCl; Py; (d) I_2; (e) R_1-NH-R_2 or amine hydrochloride, Et_3N; (f) Et_3N, H_2O

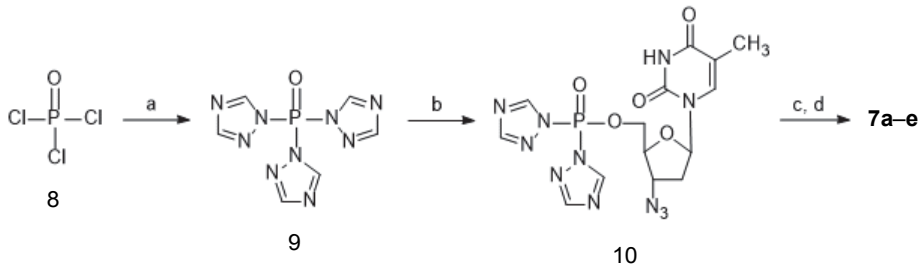

Fig. 3. Synthesis of N-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (**7a**–**e**) via di(1,2,4-triazolide) of 3′-azido-3′-deoxythymidine 5′-monophosphate (**10**); reagents and conditions: (a) 1,2,4-triazole, Et_3N, CH_3CN; (b) 3′-azido-3′-deoxythymidine (**5**); Py; (c) R_1-NH-R_2 or amine hydrochloride, Et_3N; (d) H_2O, Et_3N

species (**2**) in the initial step of reaction (Fig. 1) (Siddiqui et al., 1999). This anhydride (**2**) then undergoes hydrolysis forming phosphoramidate **3** with a free carboxyl group, which can protonate the amide group and enhance its leaving ability. Finally, phosphoramidate **3** reacts with water yielding 5 -monophosphate of AZT (**4**).

In this study, *N*-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (**7a**–**e**) were synthesized by two different routes. In the first route, 3′-azido-3′-deoxythymidine (**5**) was converted to 5′-H-phosphonate monoester of 3′-azido-3′-deoxythymidine (**6**) by reaction with diphenyl phosphite in pyridine according to a previously described procedure (Jankowska et al., 1994; Iyer et al., 2000). Then, compound **6** was reacted with trimethylsilyl chloride in pyridine to protect its free hydroxyl groups. This reaction was followed by iodine oxidation to generate reactive phosphoryl iodide intermediate (Kers et al., 1998). The intermediate formed was treated *in situ* with an appropriate amine or its hydrochloride in the presence of triethylamine to obtain products **7a**–**e**. After purification by HPLC, the yield of the products was in the range from 54 to 63% (Fig. 2). The synthesized phosphoramidates (**7a**–**e**) were identified based on their spectroscopic data (^{1}H, ^{13}C, and ^{31}P NMR, and mass spectrometry (MS)). The half-life of the phosphoramidates **7a**–**e** in aqueous solutions was found to be longer than 7 days.

In the second synthetic route (Fig. 3), the phosphorylating reagent, phosphorotri-(1,2,4-triazolide) (**9**), was first obtained by reacting phosphorus oxychloride (**8**) with 1,2,4-triazole in the presence of triethylamine (Kraszewski and Stawiński, 1980). Then, compound **9** was reacted with 3′-azido-3′-deoxythymidine to produce di(1,2,4-triazolide) of 3′-azido-3′-deoxythymidine 5′-monophosphate (**10**). The obtained product was treated *in situ* with an appropriate amine or its hydrochloride in the presence of triethylamine to synthesize the desired products **7a**–**e**. After purification by HPLC, the yield was

Table 1. *In vitro* cytotoxic activity of *N*-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (**7a**–**e**) in three human cancer cell lines (cervical cancer (HeLa), nasopharyngeal cancer (KB), and breast cancer (MCF-7)) and normal human dermal fibroblast cell line (HDF)

Compound	Cytotoxicity (IC_{50}, μM)[a] ± SD[b]				log P[c]
	HeLa	KB	MCF-7	HDF	
7a	6.21 ± 0.27	5.85 ± 0.31	5.51 ± 0.22	12.54 ± 0.49	−0.69
7b	5.19 ± 0.18	5.05 ± 0.23	4.98 ± 0.15	13.94 ± 0.32	−0.31
7c	70.23 ± 0.39	68.00 ± 0.45	65.24 ± 0.40	98.69 ± 0.56	0.24
7d	4.91 ± 0.15	4.80 ± 0.12	4.68 ± 0.11	16.78 ± 0.23	0.19
7e	7.01 ± 0.16	6.79 ± 0.21	6.38 ± 0.09	15.28 ± 0.41	0.46
AZT[d]	10.77 ± 0.66	9.77 ± 0.57	7.67 ± 0.09	14.41 ± 0.58	−0.10
FUra[d]	6.23 ± 0.46	4.84 ± 0.15	6.53 ± 0.82	7.02 ± 0.20	−1.31
ara-C[d]	3.54 ± 0.16	4.07 ± 0.08	3.82 ± 0.25	4.99 ± 0.84	−2.32

[a] IC_{50} is the compound concentration required to inhibit cell growth by 50%; [b] SD (standard deviation) of three independent experiments; [c] log P (logarithm of partition coefficient) was calculated using "log $P_{Knowwin}$" method (Pyka et al., 2006); [d] Standards: 3′-azido-3′-deoxythymidine (AZT), 5-fluorouracil (FUra), and cytarabine (ara-C)

Table 2. The calculated values of the selectivity index (SI) of the compounds **7a**–**e**

Compound	SI[a]		
	HeLa	KB	MCF-7
7a	2.02	2.14	2.28
7b	2.69	2.76	2.80
7c	1.41	1.45	1.51
7d	3.42	3.50	3.59
7e	2.16	2.25	2.40
AZT[b]	1.34	1.48	1.88
FUra[b]	1.13	1.45	1.08
ara-C[b]	1.41	1.23	1.31

[a] the selectivity index (SI) was calculated for each compound using the formula: SI = IC_{50} for normal cell line HDF/IC_{50} for respective cancer cell line; a beneficial SI > 1.0 indicates a compound with efficacy against tumor cells greater than the toxicity against normal cells; [b] standards: 3′-azido-3′-deoxythymidine (AZT), 5-fluorouracil (FUra), and cytarabine (ara-C)

35–41%. Although the yield of phosphoramidates **7a**–**e** was low, the second method was less complicated than the first, which required separate preparation of 5′-H-phosphonate monoester of 3′-azido-3′-deoxythymidine (**6**).

The synthesized *N*-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (**7a**–**e**) were evaluated for their cytotoxic activity in three human cancer cell lines (cervical cancer (HeLa), nasopharyngeal cancer (KB), and breast cancer (MCF-7)) and a normal dermal fibroblast cell line (HDF) using SRB assay (Skehan et al., 1990). The results of the evaluation of cytotoxic activity of the obtained phosphoramidates **7a**–**e** as well as the reference compounds 3′-azido-3′-deoxythymidine (AZT), 5-fluorouracil, and cytarabine are presented in Table 1. According to the results, the highest cytotoxic activity was shown by phosphoramidate **7d** with the *N*-*n*-propyl substituent (with IC_{50} in the range of 4.68–4.91 μM) in all the examined cancer cell lines. Phosphoramidate **7d** was about twofold more cytotoxic than the parent nucleoside 3′-azido-3′-deoxythymidine (AZT). In addition, a considerably higher cytotoxic activity was shown by compounds **7a**, 7**b**, and **7e**, compared to AZT. Unexpectedly, phosphoramidate **7c** with *N*-(2,2,2-trifluoroethyl) substituent was found to be the least cytotoxic among the synthesized compounds. These findings indicated that the nature of the *N*-alkyl substituent of the phosphoramidates **7a**–**e** plays an important role as a determinant of their cytotoxic activity.

To determine whether there was any correlation between the cytotoxicity and lipophilicity of the synthesized phosphoramidates, their partition coefficient (log *P*) values were calculated (Pyka et al., 2006) (Table 1). Among the phosphoramidates, compounds **7c**–**e** were found to be more lipophilic than AZT (log P = −0.10), with their log P values ranging from 0.19 to 0.46. How-

ever, linear regression analysis did not reveal any correlation between the log *P* values and the cytotoxicity of the phosphoramidates.

The cytotoxic effect of the prepared phosphoramidates was also studied in normal human dermal fibroblasts (HDF) to assess their toxicity to the normal cells (Table 1). The selectivity index (SI) of the phosphoramidates was calculated as the ratio of their IC_{50} for the normal cell line (HDF) to their IC_{50} for a respective cancer cell line (Table 2). Higher values of SI indicated a greater anticancer specificity, with compounds exhibiting SI values >3 considered to be highly selective. Among the phosphoramidates, compound **7d** showed not only a high cytotoxic activity to the cancer cells but also a low toxicity against normal fibroblast cells; its SI value was >3 for all the examined cancer cell lines (Table 2).

Conclusions

We synthesized a series of novel *N*-alkyl phosphoramidate monoesters of 3′-azido-3′-deoxythymidine (AZT) using two independent methods. In the first method, 5-H-phosphonate monoester of AZT served as a synthetic precursor, and was reacted with trimethylsilyl chloride, subsequently with iodine, and then with an appropriate amine. In the second method, AZT was phosphorylated with phosphorotri-(1,2,4-triazolide), followed by reaction with an appropriate amine and then with water in the presence of triethylamine. The obtained phosphoramidates **7a**–**e** were examined for cytotoxic activity in three human cancer cell lines (cervical cancer (HeLa), nasopharyngeal cancer (KB), and breast cancer (MCF-7)) and a normal dermal fibroblast cell line (HDF) using SRB assay. Among the phosphoramidates, the highest cytotoxic activity was demonstrated by phosphoramidate **7d** with the *N*-*n*-propyl substituent in all the investigated human cancer cells, and its activity was about twofold higher than that of the parent nucleoside (AZT). A slightly lower cytotoxic activity was shown by phosphoramidates **7a**, **7b**, and **7e**, whereas phosphoramidate **7c** with the *N*-(2,2,2-trifluoroethyl) substituent showed the least cytotoxic activity in all the cell lines used.

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